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Stimulation of Mammalian G-protein-responsive Adenylyl Cyclases by Carbon Dioxide*[S]

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Carbon dioxide is fundamental to the physiology of all organisms. There is considerable interest in the precise molecular mechanisms that organisms use to directly sense CO_2. Here we demonstrate that a mammalian recombinant G-protein-activated adenylyl cyclase and the related Rv1625c adenylyl cyclase of *Mycobacterium tuberculosis* are specifically stimulated by CO_2. Stimulation occurred at physiological concentrations of CO_2 through increased k_{cat}. CO_2 increased the affinity of enzyme for metal co-factor, but contact with metal was not necessary as CO_2 interacted directly with apoenzyme. CO_2 stimulated the activity of both G-protein-regulated adenylyl cyclases and Rv1625c *in vivo*. Activation of G-protein regulated adenylyl cyclases by CO_2 gave a corresponding increase in cAMP-response element-binding protein (CREB) phosphorylation. Comparison of the responses of the G-protein regulated adenylyl cyclases and the molecularly, and biochemically distinct mammalian soluble adenylyl cyclase revealed that whereas G-protein-regulated enzymes are responsive to CO_2, the soluble adenylyl cyclase is responsive to both CO_2 and bicarbonate ion. We have, thus, identified a signaling enzyme by which eukaryotes can directly detect and respond to fluctuating CO_2.

Inorganic carbon (C_i)[3] is central to prokaryotic and eukaryotic physiology. The predominant biologically active forms of C_i are CO_2 and HCO_3^- and their relative contributions to the total C_i pool are pH-dependent. Biological roles for CO_2 and HCO_3^- include photosynthetic carbon fixation (1), pH homeostasis (2), carbon metabolism (3), activation of virulence in pathogenic organisms (4), sperm maturation (5), and as an alarmone in *Drosophila* (6, 7).

Given its importance in biology, the identification of CO_2 responsive signaling pathways is key to understanding how organisms cope with fluctuating CO_2. Two seven transmembrane receptors, Gr21a and Gr63a, have been shown to confer CO_2 responsiveness in *Drosophila* neurons (6, 7). Guanylyl cyclase D expressing olfactory neurons also mediate sensitivity to CO_2 in mice (8). A role for cGMP-activated channels in CO_2 sensing has been observed in CO_2 avoidance behavior in *Caenorhabditis* (9, 10). Despite these impressive advances, no eukaryotic signaling enzymes unequivocally demonstrated to respond to CO_2 have been identified.

The mammalian soluble adenylyl cyclase (sAC) synthesizes the second messenger 3′,5′-cAMP and is directly stimulated by HCO_3^- (11–13). Stimulation of sAC by HCO_3^- has an unequivocal role in sperm maturation (5, 14–16). sAC is a member of the Class III family of adenylyl cyclases (ACs), a family that also includes the G-protein-regulated ACs and many examples from prokaryotic genomes (17, 18). The Class III ACs can be divided into four subclasses (a–d) based upon polymorphisms within the active site (19). sAC is a member of Class IIIb, a subclass characterized partly by replacement of a substrate binding Asp with Thr. The Class IIIa ACs include the mammalian G-protein-stimulated ACs and numerous prokaryotic examples. These have been previously assumed to be non-responsive to C_i (12).

All prokaryotic Class IIIb ACs examined to date respond to C_i including enzymes from organisms as diverse as *Anabaena* PCC 7120, *Mycobacterium tuberculosis*, *Stigmatella aurantiaca*, and *Chloroflexus aurantiacus* (20, 21). Two Class IIIb ACs, Slr1991 of *Synechocystis* PCC 6803 and CyaB1 of *Anabaena* PCC 7120, have been proven to respond to CO_2 and not HCO_3^-, giving rise to the idea of AC as a true gas-sensing molecule (22, 23). The finding that Class IIIb ACs respond to CO_2 and not HCO_3^- necessitates an examination of the assumption that G-protein-regulated ACs and related prokaryotic enzymes do not respond to C_i.

Here we demonstrate, contrary to previous work, that a recombinant G-protein-regulated AC and the Class IIIa Rv1625c AC of *M. tuberculosis* H37Rv show a pH-dependent response to C_i due to specific stimulation by CO_2 at physiologically relevant concentrations. CO_2 interacted directly with the apoprotein and modulated the activity of both the prokaryotic enzyme and G-protein-regulated AC *in vivo*. Finally, we contrasted the responses of sAC- and G-protein-regulated ACs to different species of C_i

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.
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[3] The abbreviations used are: C_i, inorganic carbon; AC, adenylyl cyclase; sAC, soluble AC; CREB, cAMP-response element-binding protein; Mes, 2-[*N*-morpholino]ethanesulfonic acid; Mops, 3-[*N*-morpholino]propanesulfonic acid; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

and propose that the mammalian cAMP signaling pathway is able to discriminate between CO_2 and HCO_3^- *in vivo*.

EXPERIMENTAL PROCEDURES

Recombinant Proteins—Rv1625c$^{204-443}$ wild type and mutant proteins, Slr1991$^{120-337}$ wild type and mutant proteins, recombinant protein corresponding to amino acids 1–469 of human sAC (truncated splice variant (13); sAC_T), recombinant protein corresponding to the first catalytic domain (amino acids 263–476; $7C_1$) of human AC type 7, and recombinant protein corresponding to the second catalytic domain (amino acids 821–1090; $2C_2$) of rat AC type 2 were expressed and purified as previously described (22, 24–27). A mixture of $7C_1$ with an excess of $2C_2$ ($7C_1$·$2C_2$) represents a catalytically active G-protein responsive AC without the transmembrane domains of the native molecule. Recombinant protein representing the short splice variant of bovine $G_s\alpha$ was purified and activated with GTPγS·Mg^{2+} as previously described (28). Single amino acid mutations were introduced by site-directed mutagenesis using appropriate primers and the appropriate wild type construct as template. Double amino acid mutations were introduced by site-directed mutagenesis using appropriate primers and the appropriate single amino acid mutant construct as template. All constructs were confirmed by double-stranded sequencing. Mutagenic primer sequences are provided in Table S1. Plasmids encoding Rv1625c$^{204-443}$ K296A and D256A mutagenic proteins were a kind gift of Joachim Schultz (25).

Adenylyl Cyclase Assays—AC assays were performed at 37 °C (Rv1625c$^{204-443}$) or 30 °C ($7C_1$·$2C_2$) in a final volume of 100 μl and contained 50 mM buffer, 2 mM [2,8-^{3}H]cAMP (150 Bq), and [α-^{32}P]ATP (25 kBq) if not stated otherwise (29). Protein concentrations were adjusted to maintain substrate utilization at <10%. The following buffers were used at pH 6.5 (Mes), pH 7.0–7.5 (Mops), and pH 8.0–8.5 (Tris-hydrochloride). Enzyme, buffer, and substrate were prepared at the appropriate pH. CO_2 was quantified by titration against NaOH. Assay pH was stable over a period of at least 40 min. For dose-response experiments, $NaHCO_3$ was added to the assay, and the CO_2 concentration was calculated using the Henderson-Hasselbalch equation, and the total salt concentration was adjusted with NaCl. All errors correspond to the S.E. If absent, errors were smaller than the symbol used to depict the data point.

Adenylyl Cyclase Assays at C_i Disequilibrium—For C_i disequilibrium assays, dissolved CO_2 was prepared by bubbling into double-distilled H_2O at 0 °C to saturation and quantified by titration against NaOH. $NaHCO_3$ and NaCl were prepared in double-distilled H_2O at 0 °C. CO_2, HCO_3^-, or Cl^- were subsequently added to the assay at 0 °C simultaneous with substrate to the required concentration. Buffer and substrate for assays were prepared at the appropriate pH and temperature for the experiment. pH changes in assays were monitored using a pH electrode (Biotrode; Hamilton) connected to a computer with a PC card (Orion Sensorlink). The pH was measured in a time-driven acquisition mode in assays identical to those used for biochemistry. All pH measurements were accurate to ± 0.02 pH units (manufacturers specifications). All errors correspond to the S.E.

CO_2 Activation of AC in Vivo—pCTXLacZ, a plasmid with lacZ expression driven from a cAMP-responsive promoter, and pQE30-Rv1625c$^{204-443}$ (25) were transformed into *Escherichia coli* M15 (pREP4). Cells were grown in Luria broth with 100 μg ml^{-1} ampicillin, 50 μg ml^{-1} kanamycin, and 5 μg ml^{-1} tetracycline at 30 °C until an A_{600} of 0.6. Rv1625c$^{204-443}$ protein production was induced with 30 μM isopropyl 1-thio-β-D-galactopyranoside for 3 h. Cells were pelleted at 4000 × *g* for 10 min and resuspended in Luria broth containing 50 mM Tris, pH 7.1. Cell suspensions were bubbled with either 10% (v/v) CO_2 in air or in air for 30 min at 30 °C. Cells were disrupted with 0.1 mg of sodium deoxycholate and 1% (v/v) toluene and mixed for 10 min at 30 °C. The lysate was made up to 50 mM sodium phosphate, pH 7.0, 0.5 mM *ortho*-nitrophenol-β-D-galactopyranoside and incubated for 15 min at 30 °C. Reactions were stopped with 2 M sodium carbonate, and absorbance was read at 420 nm. A standard curve was generated using 0–250 μM *ortho*-nitrophenol.

CO_2 Binding Assays—1 ml of 50% (v/v) Sephadex G50 in 50 mM Mes, pH 6.5 (bed volume 0.5 ml), was pre-spun at 1500 × *g* for 30 s. A freshly prepared binding reaction of 23 nmol of protein, 30 mM $NaH^{14}CO_3$, pH 6.5, and 50 mM Mes, pH 6.5, (total volume 50 μl) was immediately added and centrifuged at 1500 × *g* for 30 s, and the flow-through collected into 50 μl of 2 M NaOH. Scintillation counting was used to measure ^{14}C counts in the flow-through.

Measurement of Intracellular pH—HEK 293T cells attached to a 24-mm diameter glass coverslip were loaded with the pH-sensitive fluorescent dye 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) through exposure to 1 μM BCECF-AM (an acetoxymethyl ester derivative) for 30 min. Intracellular pH was measured by exciting a small patch of cells at 490 and 440 nm using a microspectroflourometric system and measuring emission at 535 nm. pH_i was calibrated using the high potassium nigericin method (30).

cAMP Accumulation in Vivo—HEK 293T cells were cultured in 12-well plates and labeled overnight with 1.5 μCi of [^{3}H]adenine at 80–90% confluence. Cells were washed with phosphate-buffered saline solution and incubated at the required CO_2 concentration in preincubation media (10 mM HEPES-NaOH, 117 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 11 mM glucose, 10 mM sucrose, and 2.5 mM $CaCl_2$) containing 1 mM isobutylmethylxanthine. Preincubation mixes were pre-gassed with the desired CO_2 concentration and adjusted to pH 7.0. The assay was initiated after 30 min by the addition of agonist and incubated at the required CO_2 concentration. Assays were stopped with 5% (w/v) trichloroacetic acid containing 1 mM ATP and 1 mM cAMP. Products were quantified by twin column chromatography (29). For immunoblotting, samples were harvested after treatment as above except in the absence of [^{3}H]adenine and isobutylmethylxanthine. Immunoblotting was performed using standard methodologies with anti-phospho-CREB (Ser^{133}) and anti-α-tubulin as load control.

RESULTS AND DISCUSSION

The *M. tuberculosis* H37Rv genome contains at least 15 putative ACs and one cAMP phosphodiesterase, suggesting an important role for cAMP in the physiology of *Mycobacterium*

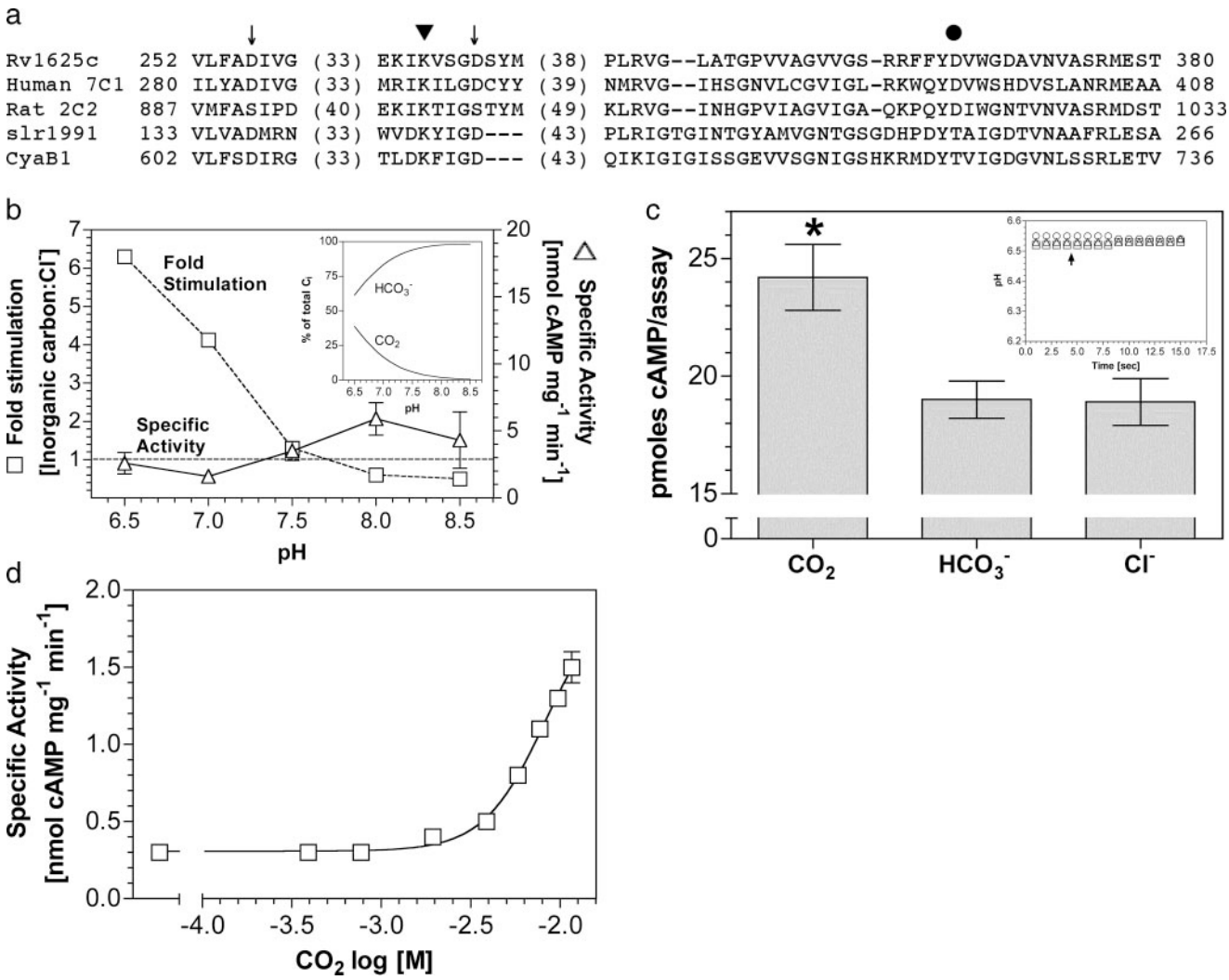

FIGURE 1. **Rv1625c is stimulated by CO_2.** *a*, alignment of the catalytic domains of Rv1625c, human AC type 7 C1 domain, rat AC type 2 C2 domain, Slr1991 of *Synechocystis*, and CyaB1 of *Anabaena*. *Numbers* denote the amino acid sequence number. *Arrows* indicate conserved metal binding aspartate residues. The *triangle* indicates the substrate binding lysine residue, and the circle is the polymorphic D/T of Class IIIa/b ACs. *b*, ratio of the specific activities of $Rv1625c^{204-443}$ when assayed in the presence of 30 mM total C_i or NaCl at various pH values (1.8 μM protein, 200 μM Mn^{2+}-ATP, $n = 8$). The *inset* shows the percentage of total C_i made up by CO_2 and HCO_3^- over the pH range tested. The figure shows specific activity in the presence of 20 mM NaCl (*triangles*, *right-hand axis*) and relative stimulation with Ci (*squares*, *left hand axis*). *c*, cAMP produced by $Rv1625c^{204-443}$ under conditions of C_i disequilibrium (36 μM $Rv1625c^{204-443}$, 0 °C, 10 s, 20 mM CO_2, 20 mM $NaHCO_3$, 20 mM NaCl, 100 mM Mes, pH 6.5, 200 μM Mn^{2+}-ATP, $n = 20$; *, $p < 0.05$). The *inset* shows a representative control experiment demonstrating that the pH was identical in all assays (*circles*, NaCl; *triangles*, $NaHCO_3$; *squares*, CO_2; *arrow*, assay start point). *d*, $Rv1625c^{204-443}$ specific activity ($n = 6$) was plotted against increasing CO_2. The assay mixture contained 433 nM protein and 200 μM Mn^{2+}-ATP, pH 6.5. The total salt concentration was adjusted to 30 mM for all data points.

(31–34). cAMP is implicated in the pathogenesis of mycobacteria, and CO_2 has been suggested as a signal to enable *Mycobacterium* to avoid phagosomal acidification (35, 36). The *Rv1625c* gene of *M. tuberculosis* encodes an enzyme consisting of six putative transmembrane helices and a single Class IIIa AC catalytic domain (25, 37). The predicted topology, therefore, resembles one-half of a mammalian G-protein-regulated AC enzyme. A further similarity arises in the active site where six key catalytic residues distributed among the two catalytic domains of the G-protein-regulated ACs are present in Rv1625c to generate a homodimeric enzyme with two active sites (Fig. 1*a*).

The Class IIIa Rv1625c AC was reported to be insensitive to C_i under experimental conditions where HCO_3^- was the predominant form of C_i. We expressed the AC domain of Rv1625c as a recombinant protein ($Rv1625c^{204-443}$) and investigated the response of enzyme to constant C_i at varying pH (Fig. 1*b*). Relative stimulation (C_i:Cl^-) varied from less than 1 at pH 8.5 (0.1 mM CO_2, 19.6 mM HCO_3^-, 0.3 mM CO_3^{2-}) to 6.3 at pH 6.5 (7.7 mM CO_2, 12.3 mM HCO_3^-). Stimulation of Rv1625c specific activity was most evident below pH 7.5, explaining a failure to previously observe a stimulation with C_i (20). A requirement for low pH to observe a response to C_i is consistent with a role for CO_2 as the activating species but may also be due to the altered protonation status of $Rv1625c^{204-443}$ limiting the ability of the enzyme to respond to HCO_3^- at higher pH. We, therefore, assayed $Rv1625c^{204-443}$ under conditions of C_i disequilibrium to determine whether CO_2 or HCO_3^- is the activating species (22, 38). AC assays performed under conditions of C_i disequilibrium exploit the fact that acquisition of the CO_2/HCO_3^- equilibrium is significantly slowed at low temperature. We defined conditions for assaying AC under conditions of dis-

TABLE 1

Kinetic parameters for Rv1625c$^{204-443}$ and 7C$_1$·2C$_2$

433 nM Rv1625c$^{204-443}$ (n = 12) or 1.1 μM 7C$_1$ and 5.8 μM 2C$_2$ (n = 9) were assayed at pH 6.5 in the presence of 20 mM total salt (7.7 mM CO_2).

Parameter	Rv1625$^{204-443}$		7C$_1$·2C$_2$	
	Cl^-	CO_2	Cl^-	CO_2
V_{max} (nmol of cAMP mg^{-1} min^{-1})	30.4 ± 0.8	76.0 ± 2.8	44.9 ± 2.8	76.4 ± 4.1
$K_{m[ATP]}$ (mM) (S.D.)	0.54 ± 0.02	1.72 ± 0.09	1.89 ± 0.25	2.04 ± 0.23
k_{cat} (s^{-1})	5.9	14.6	6.4	10.7

equilibrium by following the acquisition of the CO_2/HCO_3^- equilibrium through measuring the pH of a weakly buffered (5 mM) Mes solution on the addition of 20 mM CO_2 or $NaHCO_3$ in the presence or absence of carbonic anhydrase at 0 °C (data not shown).[4] In this manner we defined conditions for assaying AC under conditions of disequilibrium using 20 mM CO_2 or HCO_3^- as a 10-s assay period at 0 °C after the addition of C_i. Under these conditions, C_i is predominantly in the form added to the assay (CO_2 or HCO_3^-) and has not significantly advanced toward the equilibrium determined by assay pH (clamped with 100 mM Mes in the actual AC assays). Control experiments demonstrated that under the conditions used for the assay final pH was equivalent when either CO_2, HCO_3^-, or Cl^- were added, demonstrating that any observed stimulation was due to addition of C_i and not a change in assay pH (Fig. 1*c*; *inset*). C_i disequilibrium assays proved that Rv1625c$^{204-443}$ responded to CO_2 and not HCO_3^- (Fig. 1*c*). This demonstrates that a Class IIIa AC is able to respond to C_i and confirms that the response is to CO_2, as with Class IIIb ACs.

Given the similarity in response to CO_2 seen in Rv1625c$^{204-443}$ and Class IIIb ACs, we examined the kinetic parameters for Rv1625c and compared them to the Class IIIb ACs (Table 1). CO_2 stimulated Rv1625c$^{204-443}$ specific activity through an increase in k_{cat}, similar to findings with Class IIIb ACs, supporting the idea that the two subclasses share a similar mechanism of response to CO_2 (20, 22). A dose-response curve with increasing C_i revealed a 5-fold stimulation at 11.6 mM CO_2 (Fig. 1*d*). Concentrations over 12 mM caused a gradual decrease in specific activity from this peak, making an EC_{50} impossible to unambiguously calculate. Stimulation was significant to a 95% confidence interval at 1.9 mM CO_2.

Given the clear relationship between Rv1625c and the Class IIIb ACs with respect to the kinetics of activation in response to CO_2, we investigated the activation mechanism. Mutation of a key substrate determining lysine (Lys-646) in the Class IIIb CyaB1 AC of *Anabaena* ablated the response of the enzyme to CO_2 (20). We generated recombinant protein for the corresponding mutation in Rv1625c (K296A) and assessed its response to CO_2. Surprisingly, Rv1625c$^{204-443}$ K296A retained responsiveness to CO_2.[4] This finding was not unique to Rv1625c as the corresponding mutation in the Class IIIb Slr1991 AC of *Synechocystis* (K177A) was also responsive to CO_2.[4] It is plausible that the substrate determining lysine is not actually a direct site of action for CO_2, and we sought evidence for an alternative binding site. C_i has been proposed to help

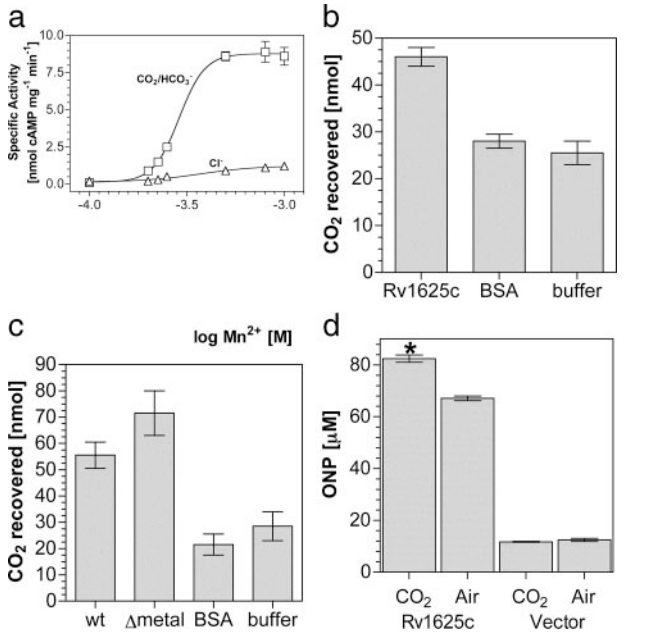

FIGURE 2. **CO_2 binds Rv1625c *in vitro* and activates *in vivo*.** *a*, Rv1625c$^{204-443}$-specific activity (n = 6) was plotted against increasing Mn^{2+}. The assay mixture contained 1.8 μM protein and 200 μM Mn^{2+}-ATP, pH 6.5, and 20 mM NaCl (*triangles*) or 20 mM $NaHCO_3$ (7.7 mM CO_2, *squares*). *b*, recovered CO_2 from a binding assay in the presence of Rv1625c$^{204-443}$, bovine serum albumin (*BSA*), or buffer alone. *c*, recovered CO_2 from a binding assay in the presence of Slr1991$^{120-337}$ wild type (*wt*), Slr1991$^{120-337}$ D137A D181A (*Δmetal*), BSA, or buffer alone. *d*, cAMP-dependent lacZ activity in *E. coli* under control (*vector*) conditions or in the presence of Rv1625c$^{204-443}$ in samples treated with air or 10% (v/v) CO_2 in air (n = 9; *, $p < 0.05$). The *y* axis denotes the concentration of *ortho*-nitrophenol (*ONP*) in the lacZ assays performed.

recruit the second metal ion to the active site of the Class IIIb CyaC AC of *Spirulina platensis* (39). Assay of Rv1625c$^{204-443}$ at varying Mn^{2+} concentrations revealed that CO_2 increased the slope of the dose response (6.6) compared with NaCl (3.0), indicating an increase in cooperativity between binding sites (Fig. 2*a*). On the basis of their findings in CyaC, Steegborn *et al.* (39) suggested that C_i interacted directly with an active site metal ion. Given our findings on Mn^{2+} recruitment for Rv1625c, we further investigated this hypothesis. Attempts to identify the metal co-factor as a site of CO_2 interaction through enzyme assay proved uninformative, and we, therefore, developed an alternative methodology.

Radiolabeled CO_2 bound to protein has been previously recovered after mixing and rapid gel filtration (40). We, therefore, performed a binding analysis to examine the requirements for CO_2 binding to enzyme. CO_2 bound Rv1625$^{204-443}$ with no requirement for metal or substrate (Fig. 2*b*). Identical results were obtained for the Class IIIb ACs Slr1991 and CyaB1.[4] Control proteins including bovine serum albumin and an alternative hexahistidine-tagged protein[4] showed recovery indistinguishable from buffer alone, indicating an absence of any specific CO_2 binding. These data would appear to eliminate a requirement for metal in the active site for CO_2 binding, but it is possible that metal co-purified with protein and remained bound to enzyme. We, therefore, investigated CO_2 binding in a mutant protein in which both metal binding aspartate residues were mutated to alanine (39, 41). The low yield of protein for Rv1625c$^{204-443}$ D256A/D300A made this experiment impossi-

[4] P. D. Townsend, P. M. Holliday, D. R. W. Hodgson, and M. J. Cann, unpublished observations.

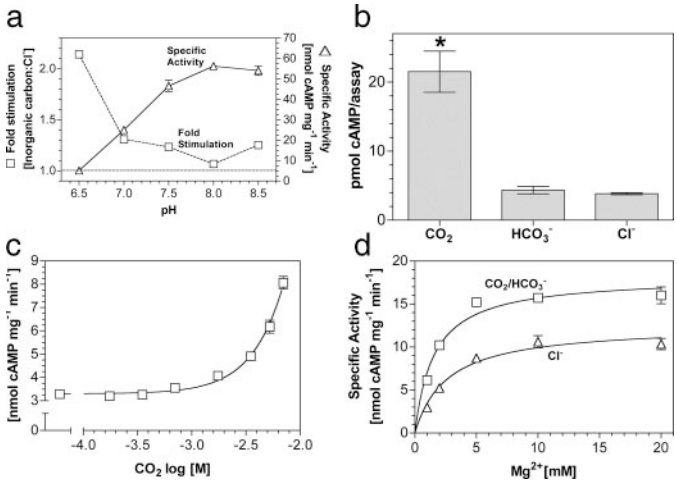

FIGURE 3. **Stimulation of a G-protein regulated AC by CO_2 *in vitro*.** *a*, ratio of the specific activities of 1.1 μM $7C_1$ and 5.8 μM $2C_2$ when assayed in the presence of 20 mM total C_i or NaCl at various pH values (500 μM Mg^{2+}-ATP, 7 μM $G\alpha_s$, $n = 6$). The figure shows specific activity in the presence of 20 mM NaCl (*triangles*; *right-hand axis*) and relative stimulation with Ci (*squares*, *left-hand axis*). *b*, cAMP produced by $7C_1{\cdot}2C_2$ under conditions of C_i disequilibrium (20 μM $7C_1$, 3.2 μM $2C_2$, 0 °C, 10 s, 20 mM CO_2/$NaHCO_3$/NaCl, 100 mM Mes, pH 6.5, 1 mM Mg^{2+}-ATP, 100 μM forskolin, $n = 6$, *, $p < 0.05$). Control experiments demonstrated that the pH was identical in all assays. *c*, $7C_1{\cdot}2C_2$ specific activity ($n = 9$) was plotted against increasing CO_2 at pH 6.5. The assay mixture contained 1.1 μM $7C_1$, 5.8 μM $2C_2$, 7 μM $G\alpha_s$, and 500 μM Mg^{2+}-ATP. The total salt concentration was adjusted to 30 mM for all data points. *d*, $7C_1{\cdot}2C_2$ specific activity ($n = 6$) was plotted against increasing Mg^{2+}. The assay mixture contained 1.1 μM $7C_1$, 5.8 μM $2C_2$, 7 μM $G\alpha_s$, and 500 μM Mg^{2+}-ATP, pH 6.5, and 20 mM NaCl (*triangles*) or 20 mM $NaHCO_3$ (7.7 mM CO_2; *squares*).

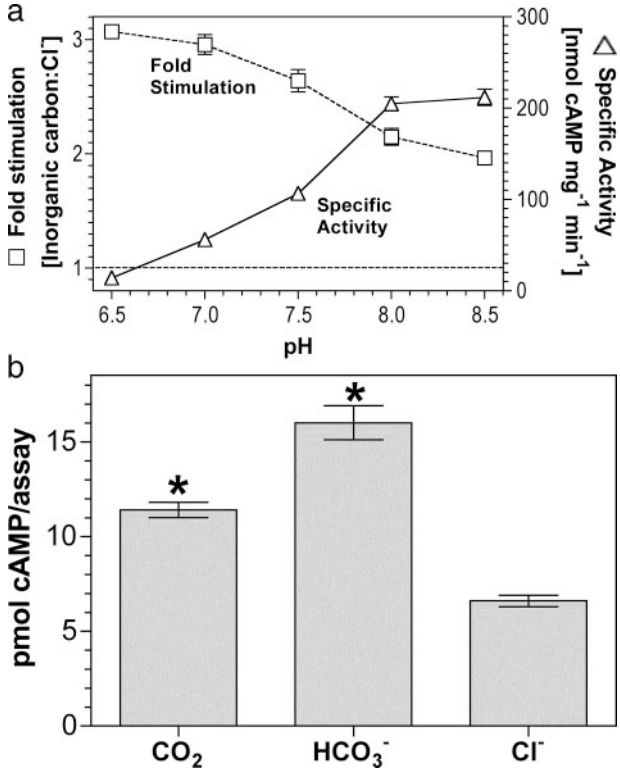

FIGURE 4. **sAC is activated by CO_2 and HCO_3^-.** *a*, 300 ng of sAC_T was assayed at 30 °C for 30 min with 0.8 mM ATP, 5 mM $MgCl_2$, and 5 mM $CaCl_2$ and either 20 mM total Ci or NaCl ($n = 4$). The figure shows specific activity in the presence of 20 mM NaCl (*triangles*; *right-hand axis*) and relative stimulation with Ci (*squares*; *left-hand axis*). *b*, 5 μg of sAC_T was assayed at 0 °C for 10 s at pH 6.4 with 0.8 mM ATP, 5 mM $MgCl_2$, and 5 mM $CaCl_2$ with either 20 mM CO_2, $NaHCO_3$, or NaCl ($n = 15$; *, $p < 0.05$).

ble for Rv1625c. We, therefore, performed the equivalent experiment in the mutant protein $Slr1991^{120-337}$ D137A/D181A (Fig. 2*c*). This confirmed that CO_2 binding occurred in the absence of metal despite the fact that the protein was catalytically inactive.[4] At physiological pH and CO_2 concentrations, only N-terminal α-amino groups and lysine side chain ϵ-amino groups are likely to be sufficiently dissociated to react with CO_2 (42). This is borne out in crystal structures in which carbamates are formed at lysine side chain ϵ-amino groups (*e.g.* Refs. 43–45). It is also possible that changes in the local environment may permit arginine to participate in a CO_2 binding site (46). Our findings indicate that the hypothesis that C_i interacts with active site metal is incorrect and that future mechanistic studies should be directed toward sites within the apoprotein.

No prokaryotic ACs have been demonstrated to respond *in vivo* to increases in CO_2/HCO_3^-. This is of obvious importance if prokaryotic ACs are to be posited as sensors of CO_2. A demonstration that Rv1625c is responsive to CO_2 *in vivo* is problematic as the numerous ACs in *Mycobacterium* make specific effects on Rv1625c impossible to distinguish. We, therefore, monitored the activity of Rv1625c expressed in *E. coli* using a cAMP responsive lacZ reporter construct as a suitable alternative. Using cAMP-driven expression of lacZ as readout, we observed a consistent increase in $Rv1625c^{204-443}$ activity at elevated CO_2 (Fig. 2*d*). LacZ produced due to endogenous cAMP was not responsive to CO_2 (Fig. 2*d*, *Vector*). As transcription of the *E. coli cya* gene (the Class I *E. coli* AC) is down-regulated by cAMP, expression of $Rv1625c^{204-443}$ likely reduced endogenous cAMP production and eliminated the possibility that our observations were due to the endogenous Cya AC (47). This demonstrates that a prokaryotic AC can be stimulated by CO_2 in an intact bacterium and, thus, fulfils a key criterion for AC as a functional CO_2 sensor in bacteria.

Building on our findings with Rv1625c, we investigated CO_2 as a stimulating ligand for a related mammalian G-protein regulated AC, an experiment of some importance as CO_2-stimulated signaling enzymes are not known in eukaryotes ($7C_1{\cdot}2C_2$; Fig. 1*a*). We investigated the response of $7C_1{\cdot}2C_2$ to 20 mM total C_i over the pH range 6.5–8.5 (Fig. 3*a*). Similar to Rv1625c, optimal stimulation of $7C_1{\cdot}2C_2$ by C_i occurred at low pH, suggesting a direct response to CO_2. Assay under conditions of C_i disequilibrium proved $7C_1{\cdot}2C_2$ was responsive to CO_2 but not HCO_3^- (Fig. 3*b*). A dose response with increasing CO_2 revealed a maximum 2–3-fold stimulation at 7 mM CO_2 (Fig. 3*c*). Specific activity decreased rather than plateaued at higher CO_2 concentrations; therefore, an $E.C._{50}$ was impossible to calculate. Stimulation was significant to a 95% confidence interval at 1.7 mM CO_2. Relative stimulation of $7C_1{\cdot}2C_2$ by CO_2 was similar when forskolin and/or $G\alpha_s$ were used to activate the enzyme, and CO_2 did not affect the affinity of $7C_1{\cdot}2C_2$ for $G\alpha_s$.[4] CO_2 increased the affinity of $7C_1{\cdot}2C_2$ for its metal co-factor (Fig. 3*d*), indicating a common mechanism of activation with Rv1625c supported by kinetic analysis (Table 1).

As sAC is proposed but not proven to respond to HCO_3^-, we investigated any overlap in specificity for C_i between sAC and the G-protein regulated ACs. sAC_T relative stimulation (C_i:Cl^-) varied from 2.0 at pH 8.5 to 3.1 at pH 6.5 (Fig. 4*a*). The

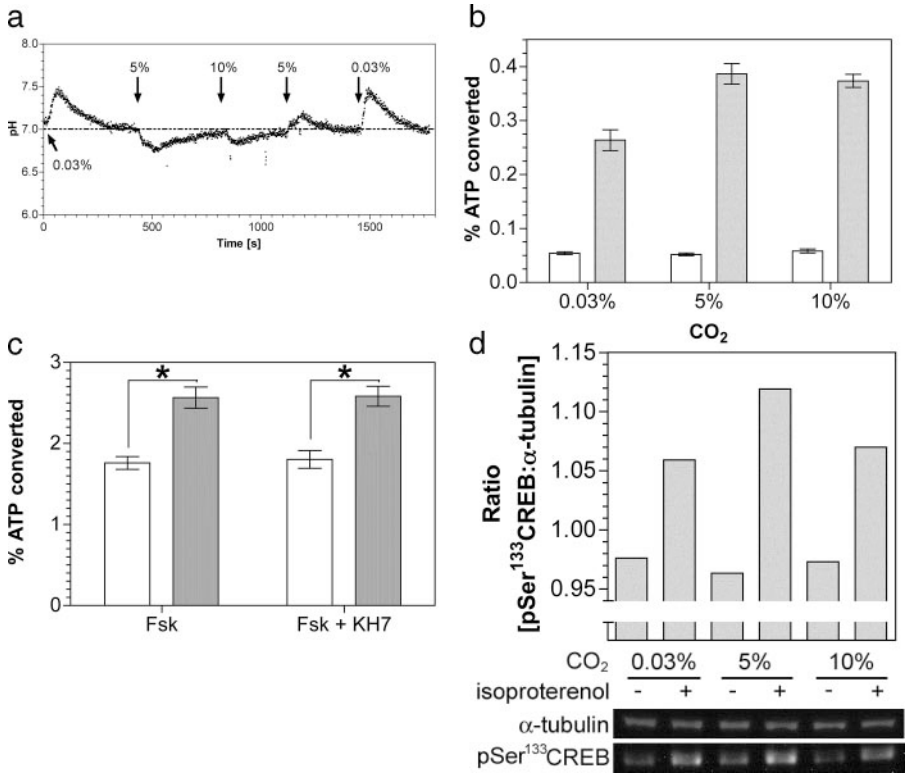

FIGURE 5. **Stimulation of a G-protein regulated AC by CO_2 *in vivo*.** *a*, monitoring of HEK 293T cell pH_i in response to changing CO_2. *b*, percentage conversion of ATP into cAMP in HEK 293T cells exposed to varying CO_2 under basal conditions (*empty bars*) or in the presence of 50 nM isoproterenol (*filled bars*) ($n = 12$). *c*, percentage conversion of ATP into cAMP in HEK 293T cells exposed to air (0.03% CO_2; *open bars*) or 5% (v/v) CO_2 (*filled bars*) with 5 μM forskolin and 1 μM KH7 ($n = 12$; *, $p < 0.05$). *d*, *lower panel* shows immunoblot of HEK 293T cell material after treatment with and without isoproterenol at varying CO_2. The *upper panel* shows the ratio of phospho-CREB:α-tubulin bands from the quantified bands.

result at pH 8.5 is consistent with a role for HCO_3^- as an activating ligand; however, the slight increase in -fold stimulation as pH is lowered suggests a response to CO_2. Under conditions of C_i disequilibrium, both CO_2 and HCO_3^- stimulated sAC_T (Fig. 4*b*).

We next investigated whether CO_2 stimulated G-protein activated cAMP signaling *in vivo*. As CO_2/HCO_3^- is a potent biological buffer, we defined conditions under which changes in internal pH (pH_i) were minimized on changing CO_2. Moving from a lower to a higher CO_2 concentration gave a transient cellular acidification and vice versa (Fig. 5*a*). Assays were, therefore, performed after allowing pH homeostasis to occur, although it is pertinent to note that G-protein-regulated ACs have been demonstrated to be offered some protection from changes in pH_i through the action of Na^+/H^+ antiporters (48). Stimulation of G-protein-activated ACs with the β-adrenergic receptor agonist isoproterenol gave an increase in cAMP accumulation in 5% (v/v) CO_2 in air *versus* air (0.03% CO_2; atmospheric concentrations of CO_2 in solution are ~0.015 μM, although the true cellular concentration is likely to be higher due to the continual production of metabolic CO_2) (Fig. 5*b*). The magnitude of this response was similar to that observed when sAC was challenged with C_i *in vivo* (12, 13). No further stimulation was observed at 10% (v/v) CO_2 as it is likely that full CO_2 activation in a precise physiological setting requires an associated carbonic anhydrase to maintain CO_2 flux (49, 50). Similar results were obtained when cAMP production was stimulated with forskolin, indicating that the stimulating effect of CO_2 does not occur upstream of AC (Fig. 5*c*). The lack of stimulation by CO_2 in the absence of agonist for G-protein-regulated AC confirmed that sAC was not the source of cAMP. The inclusion of the anti-sAC inhibitor KH7 confirmed this finding (Fig. 5*c*). We assessed downstream activation of cAMP signaling by immunoblotting using an antibody against the phosphorylated form of the cAMP-dependent protein kinase target protein cAMP-response element-binding protein (CREB). A small but significant and independently repeatable increase in phosphorylation on serine 133 of CREB in the presence of agonist was observed at 5% (v/v) CO_2 compared with 0.03% (v/v) CO_2 (Fig. 5*d*).

Our findings demonstrate that the G-protein-activated ACs are specifically CO_2-activated signaling enzymes, and this is supported by similar data in a related prokaryotic enzyme. It is possible that our findings are specific only for the $7C_1{\cdot}2C_2$ protein used in this study, as any amino acid residue(s) required for CO_2 binding may not be conserved among G-protein-regulated ACs in general. We hypothesize, however, that CO_2 regulation will be a general feature of most if not all G-protein-activated AC isoforms. A diverse range of Class IIIa, -b, and -d ACs have been demonstrated to respond to C_i. Given the extent of sequence diversity between these AC subclasses, it is unlikely that the relatively closely related G-protein-regulated ACs of Class IIIa will differ significantly in their responses to CO_2, but an examination of individual isoforms will be required to formally prove this.

Importantly, our findings overturn previous assumptions about these enzymes as non-responsive to C_i. Furthermore, we demonstrate that CO_2 interacts directly with apoprotein to stimulate metal recruitment and not through metal contact as previously proposed. We demonstrate that sAC is not the sole C_i-sensitive AC in mammals as thought and that sAC and G-protein-regulated ACs show differential sensitivity to C_i species with G-protein-regulated ACs responsive to CO_2 and sAC responsive to HCO_3^- and CO_2. Not only is the cAMP signaling pathway in its entirety, therefore, able to act as a sensing system for C_i, but different aspects of this pathway are able to discriminate between CO_2 and HCO_3^-. An interesting facet of this differential regulation is that sAC detection of C_i may be entirely independent of intracellular pH, whereas the G-protein-responsive AC signaling in response to C_i may occur predominantly under conditions of pathophysiology, *e.g.* severe respiratory acidosis or alkalosis. Some tissues are,

however, exposed to large variations in pCO_2 and have G-protein-activated cAMP signaling central to their physiology. The duodenum is exposed to a pCO_2 of up to 400 mm Hg, and the cAMP activated cystic fibrosis transmembrane conductance regulator is key to HCO_3^- secretion in this tissue (51). Acidification of the epididymal lumen with an associated low HCO_3^- concentration is essential to maintain stored spermatozoa in a quiescent state (52). It might be envisaged that these conditions would be sufficient to maintain sAC in an inactive state, but our data demonstrating that sAC is able to respond to CO_2 suggest that this cannot be the sole mechanism for keeping sAC activity switched off. A potential specific role for CO_2 signaling through G-protein-regulated ACs is evident in respiratory alkalosis. A key marker of this systemic hypocapnia is a blunted phosphaturic response to cAMP signaling through parathyroid hormone and may be explained by reduced activation of AC in response to a lowered pCO_2 (53). Further to this, CO_2 is known to regulate cAMP concentrations in the carotid body, a peripheral chemosensor, independent of pH (54). Although a role for sAC *versus* G-protein-regulated ACs in this tissue remains to be investigated, the clear role for adenosine-mediated cAMP production in the carotid body is supportive of the latter (55).

As is the case with sAC, G-protein-regulated ACs respond to CO_2 with activation of downstream signaling molecules. Changes in cAMP concentrations and CREB activation are unlikely to be an artifact of the cell culture conditions used or the composition of the assay buffer as the results obtained are clearly corroborated by data using recombinant protein. Although the percentage activation of CREB detailed in this study is far below the increase in cAMP concentrations observed, it is important to consider that HEK 293T cells may not be representative of cAMP signaling systems that are responsive to CO_2 in the organism (for example, see the hypothesized roles for cAMP signaling through CO_2 discussed). Despite these drawbacks, the system used here has successfully demonstrated that CO_2-regulated changes in hormone-activated cAMP concentrations can activate CREB phosphorylation. This is an important proof of principle even if the activation is not functional within the context of the HEK 293T cells used in this study. *In vivo* activation of AC is also conserved in a prokaryotic counterpart of the mammalian Class IIIa enzyme. Future research from these key findings should assess the role of G-protein-regulated ACs in response to fluctuating CO_2 as discussed. The cAMP signaling pathway, therefore, represents a novel signaling pathway able to directly respond to CO_2 in eukaryotes.

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